Blockade of endothelin receptors markedly reduces atherosclerosis in LDL receptor deficient mice: role of endothelin in macrophage foam cell formation

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Abstract

Objective: We evaluated the direct effects of long-term blockade of ET_A and ET_B receptors using a mixed endothelin (ET) receptor antagonist, LU224332, in the low density lipoprotein receptor (LDL-R) knockout mouse model of atherosclerosis. **Methods:** Four groups of LDL-R deficient mice were studied: control mice fed normal chow (group I); mice fed a high cholesterol (HC, 1.25%) diet alone (group II), HC fed animals treated with LU224332 (group III); and mice fed normal chow treated with the LU compound (group IV). All treatments were continued for 8 weeks at which time the animals were sacrificed and the aortae were removed and stained with oil red O. Atherosclerotic area (AA) was determined by quantitative morphometry and normalized relative to total aortic area (TA). **Results:** Cholesterol feeding resulted in a marked increase in total plasma cholesterol (-15 fold) and widespread aortic atherosclerosis (AA/TA: group I: 0.013 ± 0.007; group II: 0.33 ± 0.11; $P<0.001$). Atherosclerotic lesions were characterized by immunohistochemistry as consisting mainly of macrophages which also showed high levels of ET-1 expression. Treatment with ET antagonist significantly reduced the development of atherosclerosis (AA/TA: group III: 0.19 ± 0.07, $P<0.01$ vs. group II), without altering plasma cholesterol levels and blood pressure. The direct effect of LU224332 on macrophage activation and foam-cell formation was determined in vitro using a human macrophage cell line, THP-1. Treatment of the THP-1 cells with LU224332 significantly reduced cholesterol ester and triacylglycerol accumulation and foam-cell formation on exposure to oxidized LDL ($P<0.01$ and $P<0.05$, respectively). **Conclusion:** We conclude that a nonselective ET receptor antagonist substantially inhibited the development of atherosclerosis in a genetic model of hyperlipidemia, possibly by inhibiting macrophage foam-cell formation, suggesting a role for these agents in the treatment and prevention of atherosclerotic vascular disease. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Cholesterol; Endothelins; Macrophages; Receptors

1. Introduction

Spontaneous mutations in the low-density lipoprotein receptor (LDL-R) gene result in severe hypercholesteremia and atherosclerosis in Watanabe rabbits and rhesus monkeys [1], and represents the genetic basis of familial hypercholesteremia in humans [2]. Ishibashi et al. [3] have

produced LDL-R deficient mice by targeted disruption of this gene. On high cholesterol feeding these animals exhibited marked elevations in serum cholesterol-rich lipoprotein particles including very low density lipoprotein (VLDL), intermediate density lipoprotein (I.D.L) and LDL, associated with massive xanthomatosis and atherosclerosis in a manner similar to patients with familial hypercholesterolemia [3].

Endothelial cells normally protect against many of the

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initiating events in atherosclerosis by the production of vasodilator, antithrombotic and antiproliferative factors [4] such as nitric oxide (NO), which prevents adhesion of blood elements to the endothelium including platelets and monocytes, and inhibits migration and proliferation of medial smooth muscle cells (SMCs) [5,6]. Indeed, reduced endothelium-dependent dilation and decreased bioavailability of NO is an early feature of hyperlipidemia both in experimental models [4,7] and patients [8,9], which can be improved by administration of exogenous L-arginine, the substrate for NO generation by NO synthase (NOS) [10]. Endothelial dysfunction is characterized not only by reduced external release of vasodilator autacoids such as NO, but also by increased production of vasoconstrictor factors including endothelin-1 (ET-1) [11]. ET-1 is a 21-amino-acid peptide which, in addition to its powerful vasoconstrictor and hypertensive actions [12], has a number of other biological activities which are likely important in chronic vascular disorders. These include stimulation of cellular proliferation [13], synthesis of matrix proteins [14], and chemotactic effects on monocytes [15,16]. Several indirect lines of evidence support a role for ET-1 in the development of atherosclerosis [17]. OxLDL results in increased ET-1 expression in cultured endothelial cells [18] and circulating ET-1 levels are elevated in patients with atherosclerosis [19]. More relevant, perhaps, are the observations of increased ET-1 expression in human atherosclerotic lesions [20,21], associated with complications of atherosclerosis [22].

ET-1 transduces its biological effects through an interaction with two specific receptors. ET_A is selective for ET-1 and is found predominantly on target cells, such as vascular SMCs [23], and mediates the vasoconstrictor [24] and pro-proliferative actions of ET-1 [25]. In contrast, in the vessel wall ET_B is found mostly on the endothelial cell, and mediates the release of NO and prostacyclin [26], which serves to counteract the direct effects of ET-1 on the underlying SMCs. However, ET_B can also be found to a variable degree on SMCs [27,28] and has been described as the predominant receptor of a human monocyte/macrophage cell line [29,30].

The use of selective ET_A receptor blockers has been recently shown to reduce atherosclerosis [31,32] and improve endothelium-dependent vasodilation [32,33], possibly by unmasking ET_B-mediated NO production in response to endogenous ET-1. Whether the use of a mixed ET_A and ET_B antagonist, which would not be expected to increase vascular endothelial cell NO release, would produce a similar benefit is not certain. We hypothesized that a non-selective ET receptor blocker would reduce atherosclerosis in the LDL-R deficient mouse model by direct actions on SMCs and/or macrophages, inhibiting the proatherogenic response to increased endogenous vascular ET-1 production. We now report that LU224332, a mixed ET_A and ET_B antagonist, substantially reduced atherosclerosis in cholesterol-fed LDL-R deficient mice, and also

inhibited the uptake of OxLDL by macrophages in vitro. These data provide strong evidence for a direct role of ET-1 in atherogenesis.

2. Methods

2.1. Experimental protocol

LDL-R deficient mice in the C57BL/6J background were purchased from Jackson Laboratory. Sixty male LDL-R deficient mice were entered into the study at 22 weeks of age and were maintained on a 12-h-dark–12-h-light cycle with unrestricted access to food and water for the entire length of the experimental protocol. The use and care of LDL-R deficient mice was in accordance with the Canadian Council of Animal Care guidelines and was approved by the Animal Care and Ethics Committee of St. Michael's Hospital. Animals were assigned to four experimental groups (15 mice/group) as follows: (I) control (normal diet, no treatment); (II) high cholesterol (HC) diet without pharmacological intervention; (III) HC diet with ET antagonist treatment and (IV) ET antagonist treatment in mice receiving normal diet. All mice received their specific treatment for a period of 8 weeks before being sacrificed. The ET antagonist treatment groups received LU224332 (10 mg/kg/day) in their drinking water. This compound (a generous gift of Dr. M. Kirchengast from Knoll, Ludwigshafen, Germany) has previously been shown to exhibit equal affinity for the ET_A and ET_B receptors (ET_A: 3.5 and ET_B: 7.2 nmol/l; ratio: 2.1) [34]. To insure appropriate dosage of the ET antagonist, water intake was monitored at regular intervals and the drug dilution was adjusted accordingly. No difference in food intake, drinking patterns, or body weight was noted between animals from each group (Table 1). The HC diet consisted of 1.25% cholesterol, 7.5% (w/w) cocoa butter, 7.5% casein and 0.5% (w/w) sodium cholate. This chow preparation was shown in previous reports to promote atherogenesis [3]. After 8 weeks of treatment, mice were sacrificed and perfusion fixed with 10% formalin. The aorta were then dissected from the aortic valve to the iliac bifurcation and further fixed in 10% formalin overnight at 4°C.

Table 1
Feeding behavior and body weight variations[a]

	Group I	Group II	Group III	Group IV
Food intake (g/day)	2.4±0.3	2.0±0.5	2.1±0.6	2.4±0.6
Water intake (ml/day)	3.3±1.5	3.8±2.1	3.5±1.8	3.2±1.6
Body weight (g)	28.7±2.7	29.6±3.1	30.1±2.0	27.5±1.9

[a] Values shown are mean±S.D. No difference was noted between any experimental groups for food intake, water intake or body weight measurements by the end of the experimental protocol when subjected to one-way ANOVA with post hoc student *t*-test.

2.2. Quantification of xanthomatosis

The degree of xanthomatosis was graded according to the following scale: facial lesions: 0=none; 1=mild (snout only); 2=moderate (snout and eye lids); 3=severe (marked lesions); and limb swelling: 0=none; 1=mild/moderate (front paws only); and 2=severe (all four limbs). Addition of facial lesion and limb swelling grades represented the semiquantitative score.

2.3. Morphometry and immunohistochemistry

Aortae from each experimental group were opened longitudinally and stained with oil red O and a computer-assisted video imaging system was used to assess the extent of the atherosclerosis area (C-imaging analysis). For immunohistochemistry, the aortae of four animals from each group were divided into three regions: aortic arch, thoracic and abdominal aorta. Paraffin sections (5 μm) were cut from each region and endogenous peroxidase activity was quenched by 3% H_2O_2 in methanol for 20 min; nonspecific antibody binding was blocked with 10% goat serum in PBS for 30 min, and adjacent sections from each group were immunostained using the following antibodies: a polyclonal rabbit ET-1 antibody (Peninsula Labs., Belmont, CA, USA) at 1:150 dilution overnight at 4°C, and secondary reaction with goat anti-rabbit biotinylated antibody (1:250 dilution, Vector Labs. Burlingame, USA) for 45 min at room temperature (RT); a polyclonal rat antibody to the mouse monocyte/macrophage marker MOMA-2 (Serotec, Kidlington, Oxford, UK) at 1:100 dilution overnight at 4°C, and secondary reaction with biotinylated rabbit anti-rat IgG (1:250 dilution, Vector Laboratories) for 45 min at RT; a monoclonal mouse antibody to smooth muscle α-actin (Boehringer Manheim) at 1:100 dilution for 60 min at RT and secondary reaction with biotinylated anti-mouse IgG (1:150 dilution, Vector Laboratories) for 30 min at RT. Following incubation with the secondary antibodies, the sections were treated with streptavidin–biotin–peroxidase complexes (Vectastain ABC kit, Vector Labs.) for 30 min at RT. Diaminobenzadine was used as the peroxidase substrate and hematoxylin as the nuclear counterstain. Negative control slides were prepared by substituting preimmune serums for the primary antibody.

2.4. Cholesterol measurements

Blood was extracted by cardiac ventricular puncture in five animals in groups I, II and IV, and six for group III at the time of sacrifice and centrifuged at 1500 rpm for 10 min for plasma separation and collection. Total cholesterol was measured with an enzymatic cholesterol assay in a colorimetric procedure on a Technicon RA1000 (Bayer, Tarrytown, NY, USA).

2.5. Blood pressure measurements

In a separate experimental series, fifteen animals (five control; five HC-fed and five treated with the LU compound) were anaesthetized with an intraperitoneal injection of a mixture of xylazine (5 mg/kg, Bayer) and ketamine (50 mg/kg, Wyeth-Ayerst Canada) after 2 weeks of the representative treatments. A catheter constructed of stretched PE200 tubing (Becton Dickinson) was filled with 50 U/ml heparin in saline and was inserted into the right common carotid artery. Pulsatile blood pressure was measured using a CDXIII pressure transducer (COBE Canada) and recorded on the Biopac MP100 data acquisition system with ACKNOWLEDGE software (Biopac Systems). Animals were allowed to stabilize for 20 min after the onset of anesthesia, and then mean arterial pressure was registered continuously for 10 min and mean values were determined.

2.6. LU224332 concentrations in mouse plasma

Plasma levels of LU224332 were measured with a radioreceptor assay as previously described [35]. Briefly, 0.1 ml of plasma obtained from cardiac puncture-blood samples from animals receiving (n=7) or not receiving (n=6) the LU compound was mixed with 1 ml of methanol, thoroughly vortexed, and centrifuged for 15 min at 2800 g. The supernatant was evaporated under a stream of air. The dry residue was reconstituted in 150 μl of the binding buffer. The reaction was carried out at RT in a total volume of 200 μl; 50 μl of the radioligand (^{125}I-ET1, ≈10 000 cpm per tube) was mixed with 50 μl of the sample. The reaction was started by addition of 100 μl of porcine aortic membranes (5–7 μg protein/tube). It was terminated after 3 h by addition of 1 ml of ice-cold 5 g/l BSA in PBS, pH 7.4, followed immediately by a rapid centrifugation (3 min at 13 000 g). The supernatant was carefully aspirated, and the radioactivity of pellets was counted in an automated gamma-counter. The standard curves, constructed with 18.75 to 1200 nM of LU224332 added to normal rat plasma were linear within this range.

2.7. Cell culture

THP-1 monocyte/macrophage cell line was obtained from the American Type Tissue Culture Collection (TIB 202) and were propagated in RPMI 1640 with 10% FCS, penicillin/streptomycin (100 U/ml) at 37°C, 5% CO_2. Cells were plated at a density of 1×10^6 cells/ml in 10% FCS medium containing phorbol myristate acetate (10^{-7} M) for 72 h to induce differentiation into macrophages, and washed extensively with serum-free RPMI medium prior to incubation with or without lipoproteins as indicated for each experiment. In all experiments, cell viability exceeded 90% as determined by trypan blue exclusion.

2.8. Lipoprotein isolation and oxidation

LDL (1.019–1.069 g/ml) was obtained by density gradient ultracentrifugation [36] from plasma of fasted normolipidemic individuals. LDL (2 mg protein/ml) was subsequently dialyzed against 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA for 24 h (three buffer changes). LDL samples were sterilized by passing through an 0.22-μm filter (Millipore, Milford, MA, USA), kept at 4°C, and used within 1 week. Lipoprotein concentration was determined by the method of Lowry et al. [37] and expressed as mg/ml. Oxidation of LDL (5 mg protein/5 ml) was performed by dialysis against 5 μM $CuSO_4 \cdot 5H_2O$ in 0.1 M phosphate buffer, pH 7.4, for 12 h at 37°C in the dark.

2.9. Cellular cholesterol and triacylglycerol accumulation

THP-1 cells were incubated for 24 h with 100 μg/ml native or oxidized LDL (OxLDL) in the presence or absence of 10^{-7} M LU224332. After incubation the cells were washed once with ice cold PBS containing 0.4% BSA and twice with PBS alone. Cells were scraped from the culture flask into PBS and sonicated. The cellular lipids were extracted with chloroform–methanol (2:1, v/v). The lipid extract was digested with phospholipase C (Clostridium welchii; Sigma) as previously described [38]. The reaction mixture was extracted with chloroform–methanol (2:1, v/v) containing 100 μg tridecanoylglycerol as internal standard. The lipid extracts were then reacted for 30 min at 20°C with Sylon BFT (Sigma) plus one part dry pyridine. This procedure converts the free fatty acids into silyl esters and the free sterols, diacylglycerols and ceramides into silyl ethers, leaving the cholesteryl esters and triacylglycerols unmodified. The free cholesterol, cholesterol esters and triacylglycerols were quantified using a nonpolar capillary column as previously described [39].

2.10. Data analysis

Statistical differences between groups were evaluated using the one-way ANOVA test with post hoc student t-test where appropriate. For semiquantitative scoring of xanthoma, the statistical difference between groups was evaluated using the Mann–Whitney test. Data are presented as mean±S.D. unless otherwise indicated. A value of $P < 0.05$ was considered significant.

3. Results

Cholesterol-fed animals accumulated foam-cells along the inner curvature of the aortic arch and throughout the descending aortae, leading to the formation of fibro-fatty plaques at 8 weeks of treatment (Fig. 1b, d and f).

Histological examination revealed that the atherosclerotic plaques contained a necrotic core with cholesterol crystals covered by a thin fibrous cap. Occasional SMCs could be identified in the plaque area and fibrous cap by immunostaining with an antibody against α-actin (Fig. 1b), however, α-actin positive cells were mostly restricted to the medial layer of the aortae (Fig. 1a and b). Immunostaining with monocyte/macrophage specific antibody (MOMA-2) showed little or no staining in animals receiving normal chow (Fig. 1c), whereas the majority of cells within the intimal lesion of HC fed animals were MOMA-2 positive (Fig. 1d). In animals receiving normal chow, ET-1 staining was restricted to endothelial cells (Fig. 1e), whereas ET-1 was predominantly located to macrophage rich intimal aortic lesions of HC treated animals, consistent with previous reports [15,21] (Fig. 1f).

The degree of xanthomatosis, derived using a semiquantitative grading system, is presented in Fig. 2A. In LDL-R knockout mice fed a normal chow for 8 weeks (Fig. 2, group I), no xanthomatous lesions were observed. In contrast, in the cholesterol-fed LDL-R deficient mice (group II) xanthomatous lesions of the face, ventral surface of the trunk and swelling of the extremities began to appear at 6 weeks and were present in all animals by 8 weeks [xanthomatosis score (XS) of 4.0±0.6 (median±S.D.) Fig. 2]. In the cholesterol-fed LDL-R deficient mice treated with ET antagonist (group III), significantly fewer xanthomatous lesions were apparent at 8 weeks [XS: 1.5±0.5 (median±S.D.) Fig. 2]. LDL-R deficient mice fed 1.25% cholesterol were severely hyperlipidemic with mean plasma cholesterol levels 15-fold higher than normal chow-fed animals (group I: 4.8±0.6 mM vs. group II: 65.6±6.5 mM; $P < 0.001$). Treatment of cholesterol-fed LDL-R deficient mice with the ET antagonist did not alter plasma lipid levels (group III: 66.6±5.1 mM) (Fig. 2B). As well, arterial blood pressure was not significantly different in animals fed normal or HC diets (78±7 and 78±3 mmHg, respectively), either with or without treatment with the ET antagonist for 15 days (74±7 and 78±3 mmHg, respectively) (five animals in each group). These results are consistent with previous reports using endothelin antagonist in mice [32] and other normotensive animal models [40]. Treatment with LU224332 (10 mg/kg/day for 2 weeks) resulted in measurable plasma levels of the ET antagonist (708±357 nmol/l), which was well in excess of the K_i for both ET receptors (see Methods).

The extent of aortic lipid deposition was visualised by oil red O staining (Fig. 3A) and quantified by computer assisted morphometry (Fig. 3B). Extensive atherosclerosis was seen in the HC diet group (group II), whereas only minimal lipid deposition was found in animals receiving normal mouse chow mainly at the bifurcations of great vessels (group I). LU224332 treatment (group III) significantly reduced the extent of atherosclerotic involvement in the aortae by almost 45% (Fig. 3B, $P < 0.01$).
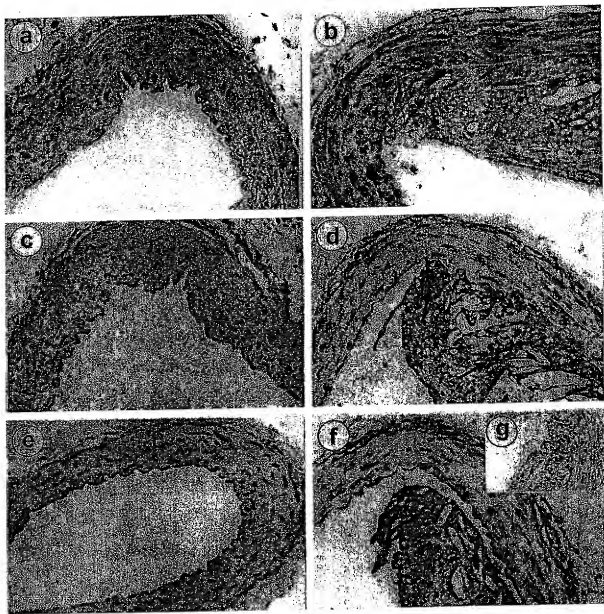
Fig. 1. Photomicrographs show representative sections of thoracic aorta from LDL-R deficient mice fed normal chow (groups I: a, c and e) or a high cholesterol diet (group II: b, d, f and g). Immunostaining for SMCs using an α-actin antibody revealed a similar pattern of staining in both normal chow (a) and high cholesterol (b) fed animals, largely restricted to the medial layer of the vessels with only partial staining in the atherosclerotic lesion. In contrast, immunostaining with MOMA-2 revealed an absence of macrophages in normal chow fed animals (c) with a very dense accumulation of macrophages in the lesions of high cholesterol fed animals (d). Immunostaining for ET-1 on sequential sections revealed expression of this peptide limited to endothelial cells of normal chow-fed animals (e), with marked ET-1 staining in the HC animals (f) predominantly located to the intimal macrophage rich lesions. Negative control slides were prepared by substituting preimmune rabbit serum for the primary antibody in a section from group II (g).

In order to study the direct effect of endothelin receptor blockade on macrophage lipid accumulation, THP-1 human macrophages were incubated with 100 μg/ml of native LDL (nLDL) or Ox LDL, in the presence or absence of LU224332 (10^{-7} M). After 24 h, cellular cholesteryl ester (CE) and triacylglycerol (TG) were quantified as described in Methods. Treatment of cells with Ox LDL resulted in 3-fold increase in CE and TG levels compared to nLDL alone ($P<0.01$ and $P<0.05$, respectively; Fig. 4A). The addition of LU224332 completely prevented macrophage CE and reduced TG deposition induced by Ox LDL ($P<0.01$ and $P<0.05$, respectively; Fig. 4B), reducing macrophage lipid accumulation to levels not different from nLDL alone.
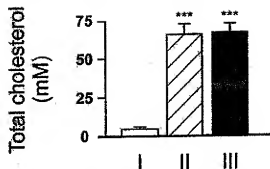
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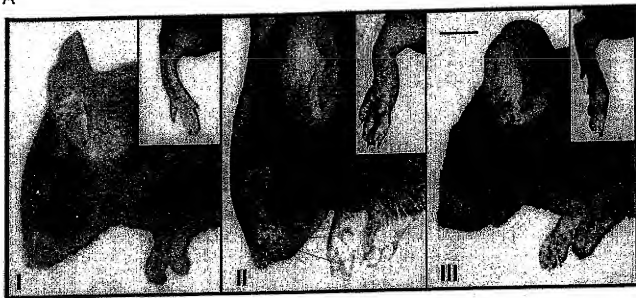
B

Fig. 2. Animals on regular chow diet, (group I), did not develop xanthomas. In contrast, mice fed high cholesterol (HC) diet alone, group II, developed facial xanthomatous lesions and marked swelling of the paws by the end of the experimental protocol. The LDL-R deficient mice receiving supplemental endothelin antagonist (LU224332) together with HC diet, (group III), showed much reduced facial xanthomatous and minimal swelling of the extremities (A). The mean xanthomatosis score for experimental groups II and III are presented in the results section. Average total plasma cholesterol values (mM) in groups I, II and III are shown in (B).

4. Discussion

The results of the present study demonstrate an important anti-atherosclerotic effect of a non-selective ET receptor antagonist in a model of homozygous familial hypercholesterolemia, the LDL receptor (LDL-R) deficient mouse. In addition to preventing atherosclerosis, treatment with the ET antagonist significantly reduced xanthoma formation without affecting total cholesterol levels or arterial pressure. These results support the hypothesis that the ET system contributes directly to the pathogenesis of atherosclerosis and that ET blockers may have therapeutic utility in the treatment of this vascular disorder.

In the vessel wall, the ET_A receptor is located primarily on SMCs, whereas the ET_B subtype is found mainly on the endothelial layer, infiltrating macrophages [29] and to a variable extent SMCs [28]. Although ET_A may mediate many of the effects of ET-1 that are likely relevant to atherosclerosis, the presence of the ET_B receptors on macrophages and its up regulation on SMCs of vascular lesions [27], suggest that this receptor subtype may contribute importantly to the pathogenesis of atherosclerosis as well. In fact, a recent report has suggested that accumulation of foamy macrophages and T lymphocytes in the fibrous plaque may modulate the switching of ET receptor subtypes from ET_A to ET_B in SMCs [41].
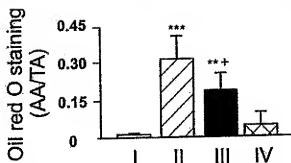
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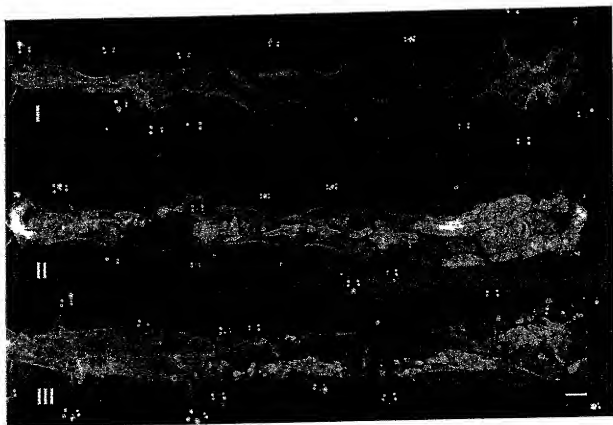
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Fig. 3. Representative oil red O staining on the full length luminal surface of aortas from LDL-R deficient mice. Extensive lipid deposition could be seen in the HC diet group (group II), while only minimal lipid deposition was found in animals receiving normal mouse chow (group I). Aortas from LU224332 treated animals (group III) showed reduced aortic atherosclerosis (A). Mean values for atherosclerotic area relative to total aortic luminal surface are shown in panel B ($n=6$, group I; $n=9$, group II and III; $n=5$, group IV). Asterisks indicate statistical difference versus group I using the one-way ANOVA with post-hoc Student t-test (**, $P<0.01$; ***, $P<0.001$). The plus sign indicates a statistical difference versus group II using the one-way ANOVA with post-hoc Student t-test (+, $P<0.01$).

Cultured rat peritoneal macrophages have been described to express nearly exclusively ET_B receptors [42] whereas both ET_A and ET_B receptors have been demonstrated by in situ hybridization on macrophages in the early inflammatory intimal lesion of hyperlipidemic hamsters [31].

In contrast, stimulation of ET_B receptors on the endothelial cells releases vasodilators, such as NO, which may protect against atherosclerosis [43]. Kowala et al. [31] previously reported that an ET_A selective antagonist re-

duced fatty-streak formation in a hamster model of early atherosclerosis. However, to some extent this effect might have been due to a lipid lowering action of certain ET antagonists [31,44]. Recently, Barton et al. [32] reported that another ET_A selective antagonist reduced atherosclerosis in the apoE-deficient mouse model of atherosclerosis, further supporting an important role for ET-1 in this disease. This was associated with a marked improvement in endothelium-dependent dilation and increased nitrate/
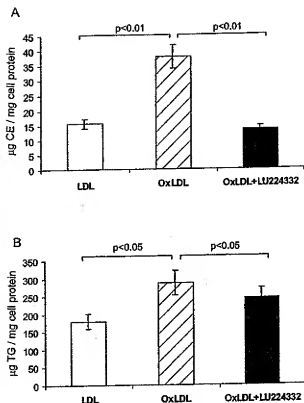
A

B

Fig. 4. Mean values±S.D. ($n=4$) for cellular cholesterol ester (CE; A) and triacylglycerol (TG; B) accumulation in THP-1 cell line in presence of nLDL or OxLDL with or without LU224332 for 24 h. LU224332 prevented CE and TG uptake induced by OxLDL ($P<0.01$ and $P<0.05$, respectively).

nitrite levels in the blood [32], likely as a result of selective ET_A blockade which spares the endothelial ET_B receptor. Therefore, it is possible that an increase in endothelial NO production may have contributed indirectly to the anti-atherogenic effects of ET_A blockade in these studies. It is well established that other strategies to increase endothelial NO release, i.e. L-arginine supplementation [45,46], and angiotensin converting enzyme inhibition [47,48] reduce atherosclerosis in a variety of animal models. In the present study a balanced ET_A and ET_B receptor antagonist was used, which would not be expected to favorably alter the balance of endothelial versus smooth muscle ET receptor activation. Indeed, it could be argued that blockade of endothelial ET_B receptor with this compound would be counterproductive and could reduce the overall beneficial effect of the ET antagonist in atherosclerosis models. Nonetheless, a marked reduction in atherosclerosis and xanthomatosis was seen with LU224332 in the absence of any changes in plasma lipids, which may be ascribed to direct effects of ET-1 on the cellular events leading to the initiating and/or progression of atherosclerosis. However, we cannot exclude the possibility that mixed ET blockade may have resulted in

improvement in endothelial function by an indirect mechanism. Increased NO production has been previously reported with both selective and non-selective ET antagonists in the rat Langerdorff heart model [49], possibly due to increased coronary flow and therefore intimal shear forces [49].

In addition to its potent vasoconstrictor effects, ET-1 has a number of biological activities, which might contribute directly to the morphological changes characteristic of atherosclerosis. Endothelin-1 is a co-mitogen for vascular SMCs [13], and can act in concert with other well-characterized growth factors, such as PDGF, which are believed to initiate and maintain cell proliferation in the atheromatous [17]. ET-1 is also a powerful stimulus for secretion of collagen [14] and other matrix components which represent a major constituent of the atherosclerotic lesion. Therefore the inhibition of ET-1 action on atheromatous SMCs may be critical in the anti-atherosclerotic effects of LU224332. As well, ET-1 may also contribute to the recruitment of monocytes into the developing intimal lesion either directly [15] or indirectly by increasing MCP-1 [16]. Macrophages play a key role in the pathogenesis of atherosclerosis [30]. The marked up-regulation of expression of ET-1 in macrophages seen in this and other studies also suggest that this peptide may contribute to chronic inflammatory changes in this disease.

ET-1 has been shown to increase the release of inflammatory cytokines from macrophages [50,51]. In turn, cytokines such as TNFα, IL-1 and IL-6 have been shown to increase ET-1 production by macrophages [52]. Thus ET-1 may serve to amplify and sustain macrophage activation in the developing atheromatous [51]. Interruption of this positive feedback pathway is a potential mechanism by which ET receptor antagonists may reduce the progression of atherosclerosis in addition to its effects on SMC proliferation and matrix secretion. In support of this, a marked decrease in xanthomas formation, a non-vascular lesion that is dependent on macrophage activation [3] was also observed in LDL-R deficient mice treated with the ET antagonist. Further evidence in favor of a direct effect of ET-1 on macrophage foam-cell formation was provided by in vitro studies using the human THP-1 monocyte-macrophage cell line. These cells differentiate into macrophages on exposure to phorbol ester, in which state they have previously been characterized to express predominantly the ET_B receptor [29]. The ability of the LU224332 compound to largely prevent cholesterol ester and triacylglycerol accumulation in these cells on exposure to Ox LDL is consistent with a crucial role for endogenous ET-1 in macrophage activation and foam-cell formation.

In summary, nonselective inhibition of ET receptors with LU224332 reduced atherosclerosis and xanthomatosis independently of any change in lipid levels. Prominent ET-1 expression in macrophage-rich atherosclerotic lesions observed in vivo, together with the ability of the ET receptor antagonist to directly reduce macrophage lipid

accumulation in vitro, point to a role for ET-1 in foam-cell formation. Thus, antagonism of the ET system may provide a new pharmacological approach to reduce the vessel wall response to chronic injury induced by hyperlipidemia, and thereby inhibit intimal lesion formation and progression of atherosclerosis.

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